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RICIN TOXICITY IN BALB/C 3T3 CELLS: PEPTIDE BIOMARKERS OF EXPOSURE

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14. ABSTRACT

This report is required for the U.S. Army Edgewood Chemical Biological Center In-House Laboratory Independent Research project, "Cell Toxicity by NMR and Mass Spectrometry." Nuclear magnetic resonance and/or mass spectrometry (MS) in combination with chemometrics analysis can yield physiological details in the form of metabolome or proteome biomarker information. We have analyzed trypsinized cellular protein extracts from BALB/c 3T3 murine fibroblasts dosed with vehicle control and with three ricin concentrations. At the time of sample collection (48 h post dosing), only attached cells were harvested. Adjustment was made for cell concentration in preparing samples for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Analysis of the LC-MS/MS data to determine peptide sequences, followed by statistical filtering, has shown that at least one 3T3 peptide biomarker exists for each biological cell group (vehicle control and 3 ricin dose levels). These peptides correspond to particular proteins expressed by the 3T3 cells. Identification of the relevant proteins, followed by metabolic pathway analysis to determine the pathways in which those proteins are involved, may reveal the survival mechanism for cells dosed at low levels, which in turn will provide insight into the related 3T3 physiological changes required to overcome ricin dosing.

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PREFACE

The work described in this report was authorized under Project No. 61110191A00, In-House Laboratory Independent Research. This work was started in October 2009 and completed in September 2010.

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RICIN TOXICITY IN BALB/C 3T3 CELLS: PEPTIDE BIOMARKERS OF EXPOSURE

1. INTRODUCTION

Traditional cell toxicity assays lead to an IC_{50} or EC_{50} value based on the determination of cell death. However, additional information is desirable for understanding a toxin's total effects. Nuclear magnetic resonance (NMR) and/or mass spectrometry (MS) in combination with chemometrics analysis can yield physiological details in the form of metabolome or proteome biomarker information. We hypothesize that protein biomarkers in the form of dose dependent differences in protein expression occur upon ricin exposure, and that these can be identified using liquid-chromatography tandem mass spectrometry (LC-MS/MS) based proteomics methods. Here, we report on a study to explore this hypothesis for LC-MS/MS with ricin dosed BALB/c 3T3 murine fibroblasts by the analysis of tryptic peptide digests of the cellular proteins.

2. METHODS

Note: Ricin is extremely toxic. Its use is controlled under the Biological Select Agents and Toxins Program in the United States by the Centers for Disease Control and Prevention (Atlanta, GA). Handling of ricin should follow strict safety procedures determined in collaboration with the safety office of the research laboratory's organization.

2.1 Ricin Preparation.

Ricin communis agglutinin II (ricin, Vector Laboratories, Burlingame, CA) was dialyzed into 10 mM sodium phosphate buffer (pH 7.0, PB) over a period of ~24 h with gentle stirring using three volumes of ~600 mL PB. Dialysis was carried out on ice using regenerated cellulose or cellulose ester Dispodialyzers® (Spectrum Laboratories, Rancho Dominguez, CA) with 5000 or 8000 molecular weight cutoff. Dialyzed ricin was stored at 0 - 4 °C. The dialyzed ricin concentration was determined at 25 °C by ultraviolet (UV) absorbance using either a JASCO Model J-810 Spectropolarimeter (JASCO Analytical Instruments, Easton, MD) equipped with a PTC-423S Peltier thermoelectric temperature control system or a NanoDrop® ND-1000 spectrophotometer (NanoDrop® Technologies, Inc., Thermo Fisher Scientific, Waltham, MA). For the JASCO method, 15 absorbance measurements on one aliquot of the solvent blank solution (no protein) were recorded at intervals of 1 s and averaged. Fifteen measurements were then recorded on one aliquot of the ricin sample and averaged. The blank average was subtracted from the sample average. The concentration was calculated using Beer's Law with $E^{0.1\%}_{280nm} = 1.4$.[1] For the Nanodrop® method, blank-subtracted measurements were recorded on an aliquot of the ricin sample. The concentration was calculated using Beer's Law with $E^{0.1\%}_{280nm} = 1.4$.[1] The Nanodrop® measurement procedure and concentration calculation procedure were carried out three times, each using fresh aliquots of blank and ricin sample, and the concentration results averaged. The results of the two procedures were within error of each other. The dialyzed ricin was sterile filtered prior to use with the cell cultures and the concentration verified by the Nanodrop® method.

2.2 Ricin Exposure of BALB/c 3T3 Murine Fibroblasts.

The fibroblasts (CCL-163 American Type Culture Collection [ATCC], Manassas, VA) were passaged a minimum of three times after thawing prior to testing. Cell culture flasks (75 cm^2) were seeded at 8 x 10^3 cells/cm^2 and maintained in culture at 37 °C in a humidified atmosphere of 5% CO_2 in air for 24 h prior to treatment. Exposures were performed 24 ± 2 h after seeding the 75 cm^2 flasks. Three concentrations of ricin were prepared by diluting the dialyzed ricin in cell culture medium. The concentrations corresponded to IC_{20}, IC_{50}, and IC_{80} concentrations previously determined by Neutral Red Uptake (NRU) assay.[2,3] Six replicate sets of flasks (two flasks per set) were prepared per ricin concentration. In addition, flasks were prepared containing untreated cells (Vehicle Controls, VC), and containing media with and without ricin. We will refer to the four groups of cells treated identically (VC and three ricin dose levels) as biological cell groups. After ricin exposure, the flasks were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air for another 48 h.

Cell harvesting was carried out 48 h ± 0.5 h post-exposure. Medium from each set of flasks (two flasks per concentration of ricin and vehicle controls) was removed. The attached cells remaining in the flasks were rinsed twice with Hank's Balanced Salt Solution (HBSS) and then incubated at room temperature for 3 min with trypsin-versine (Product # 17-161E, Lonza Walkersville, Inc., Walkersville, MD). The trypsin-versine was neutralized with the addition of 37 °C Routine Culture Medium (RCM) containing serum. The cells were removed from the flasks and centrifuged for 5 min at 1200 rpm. The supernatant was then discarded, and the pellet was resuspended in 4 mL of 37 °C PBS. A 700 µL sample was removed for cell counts and viability analysis using a ViCell-XR (Beckman Coulter, Brea, CA). The remaining cells were centrifuged for 5 min at 1200 rpm. The supernatant was discarded. The cell pellet was resuspended in 1 mL of 37 °C PBS and then frozen at -80 °C.

2.3 Liquid-Chromatography/Mass Spectrometry Sample Preparation.

The cell samples were thawed and lysed by ultrasonication (25 s on, 5 s off, 4 min total) using a Branson Digital Sonifier® (Danbury, CT). The lysate was centrifuged at 14,000 rpm for 20 min at 10 °C using a Beckman GS-15R centrifuge and F2402H Rotor. The supernatant was transferred to a Microcon® YM-3 filter unit (Millipore, Billerica, MA) and centrifuged at 14,000 rpm and 10 °C (500 µL for 30 min x 2, total volume of 1.00 mL). The filtrate was stored at -20 °C for other analyses. The cellular proteins in the retentate were denatured overnight at 37 °C with 300 µL of 7.2 M urea and 3 µg/mL dithiothreitol in 100 mM ammonium bicarbonate (ABC). The urea was removed by centrifugation (14,000 rpm, 30 min, RT), and the retentate was washed with 200 µL ABC followed by centrifugation using an Eppendorf centrifuge (5415C with rotor F-45-18-11 or 5415D with rotor F-45-24-11, Eppendorf North America, Westbury, NY) at 14,000 rpm for 30 min (RT). The filter unit was then transferred to a new receptor tube, and the proteins in the retentate were digested at 37 °C for 7 h with 5 µL sequencing grade trypsin (Product # 511A, Promega, Madison, WI) in 10 µL acetonitrile and 235 µL ABC. The tryptic peptides were isolated by centrifuging at 14,000 rpm, 15 min, RT (Eppendorf 5415C or D). The filtrate containing the tryptic peptides was stored at -20 °C until analysis.

2.4 Liquid-Chromatography/Mass Spectrometry Experiments.

A dilution scheme was prepared for the tryptic peptide samples based on the total cell counts in the 700 µL samples (see Section 2.2), and on a final desired total volume of 40 µL for each MS sample. A dilution factor was calculated for each tryptic peptide sample by dividing the total cell count for that sample into the total cell count for the sample having the highest total cell count. The diluent was a solution of 95% H_2O and 5% acetonitrile.

Of the 40 µL dilution volume prepared for each MS sample, 27 µL was expected to be injected. However, the instrument could only accommodate a 25 µL maximum injection volume. Thus, 25 µL of each MS sample was injected into a Thermo Electron Corporation Finnigan Surveyor HPLC (Thermo Scientific, Waltham, MA), and the peptides were separated using a 0.1 x 150 mm C_{18} Hypersil GOLD KAPPA column (5 µ particle size, 175 Å pore size, Fisher Scientific International, Pittsburgh, PA) with a linear gradient of 20 to 80% organic phase (100% acetonitrile, 0.1% formic acid). The column was connected to a Finnigan LTQ tandem ion trap MS fitted with a nanospray ESI source operated at 1.82 kV with a collision energy of 25 V. A data-dependent mode and scan range of *m/z* 300-2000 were used. The full mass spectra were collected, followed by MS on the resulting five most intense ions.

The MS samples were analyzed in a semi-random order, with six groups back-to-back and each group containing four samples (one from each ricin dose level). In addition, at least three different replicate sets were represented per group. For example, the third group included peptide samples run in the following order: IC_{50}, replicate set 3; IC_{20}, replicate set 5; VC replicate set 2; IC_{80}, replicate set 2. The mass spectrometer operator was "blind" to sample identities.

2.5 Proteomics Analysis.

A protein database was constructed in a FASTA format using the annotated proteome sequences from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov, accessed July 27, 2010). For this task, an in-house PERL (http://www.activatestate.com/Products/ActivePerl, accessed July 27, 2010) program was used to automatically download annotated human, bovine, rat, and mouse proteome sequences from the NCBI. The database was constructed by translating putative protein-encoding genes and contains amino acid sequences of potential tryptic peptides obtained by the *in silico* digestion of all proteins, assuming up to two missed cleavages. The acquired mass spectra were searched against this database with the SEQUEST algorithm (Thermo Scientific). The SEQUEST thresholds for searching the product ion mass spectra were Xcorr, deltaCn, Sp, RSp, and deltampep. These parameters provide a uniform matching score for all candidate peptides.

The raw results obtained from SEQUEST were used as input into ProteoIQ (NuSep, Bogart, GA) for label-free statistical filtering using the spectral count method[4] to identify those peptides having sequences corresponding to amino acid stretches in known proteins. The data was analyzed using the following parameters: 1) peptide length = 5 amino acids, (2) minimum Xcorr = 1, (3) minimum peptide probability = 0.05, and (4) minimum protein probability = 0.5. No charged state filter was applied (all charged states used). No decoy data base was applied.

3. RESULTS AND DISCUSSION

The LC-MS/MS experiments were carried out on 24 cellular protein extract samples as described above and summarized in Table 1. Typical LC-MS/MS data for a single peptide sample is shown in the Figure.

Table 1. Summary of Tryptic Peptide Samples Analyzed by LC-MS/MS

Biological Cell Group	Ricin Concentration (μg/mL)	# of Replicate Samples	Average Total Cell Count in 700 μL Suspension
Vehicle Control Cells	---0---	6	1.37×10^{6}
Cells dosed w/ ricin at IC_{20}	9.54×10^{-04}	6	0.78×10^{6}
Cells dosed w/ ricin at IC_{50}	2.15×10^{-03}	6	0.41×10^{6}
Cells dosed w/ ricin at IC_{80}	5.97×10^{-03}	6	0.15×10^{6}
Total	----	24	

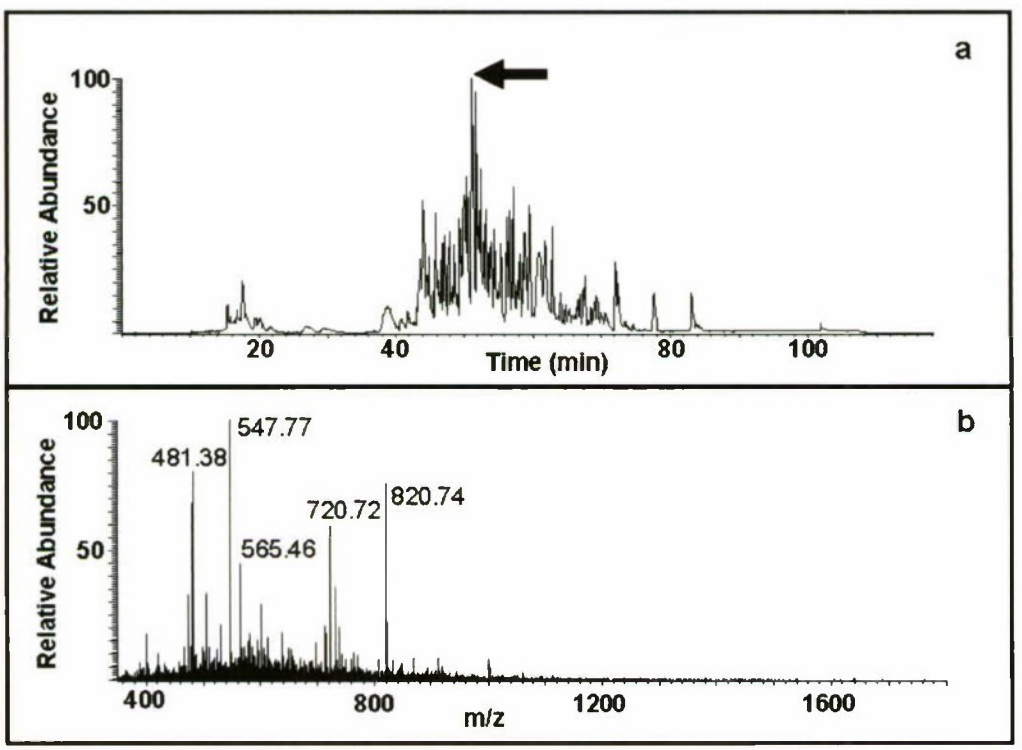

Figure. LC-MS Data from Extracted Cellular Proteins. a) Total ion chromatogram (The arrow marks the peak having a 51-min retention time.), and b) Full MS spectrum for LC peak with 51-min retention time.

Statistical filtering of the MS data (SEQUEST analysis followed by ProteoIQ analysis) identifies amino acid sequences ("hits") that correspond to observed m/z values for the peptides in a sample and provides a score and probability for each peptide. The score, calculated by SEQUEST, indicates how well the observed spectrum matches the theoretical spectrum for a given peptide sequence. The probability, calculated by ProteoIQ, indicates the likelihood that the peptide sequence is correctly determined based on the observed mass data. Using a minimum peptide probability of 0.05, more than 14,000 peptides total were identified for the tryptic peptide samples from the biological cell groups (vehicle control and three ricin dose levels). Table 2 contains the results for some representative peptide sequences. Further filtering was carried out with a minimum peptide probability of 0.95, resulting in ~3600 hits. The ~3600 hits represent 968 different peptide sequences that were each identified in one or more spectra from the 24 samples analyzed. Eleven percent of the peptides were observed at all dose levels, and we focus on these peptides for this report. These peptides are expected to be mapped to proteins expressed by all biological cell groups. Table 3 contains a spectral count summary for the peptides having a total spectral count of 24 or higher. The total spectral count value represents the total number of times the peptide was observed for all samples. For a given biological cell group, a peptide having a spectral count considerably higher or lower for that biological cell group as compared to other biological cell groups would represent a biomarker peptide. In Table 3, for a given peptide, the spectral count percentage is highlighted if it is at least 10 percentage points higher for a particular biological cell group than for the other biological cell groups. The highlighted peptides thus represent peptide biomarkers. At least one peptide biomarker is noted for each biological cell group. This analysis indicates the high likelihood of success in identifying protein biomarkers to which these peptides correspond. Completion of protein identification and metabolic pathway mapping will reveal the protein biomarkers and the biochemical pathways affected, respectfully. The resulting information will further our understanding of the physiological changes that occur in BALB/c 3T3 cells with ricin dose.

4. CONCLUSIONS

Here we have analyzed cellular protein extracts from BALB/c 3T3 murine fibroblasts dosed with ricin. The cell concentration per dose was constant at the initial time of dosing. At the time of sample collection (48 h post dosing) only attached cells were harvested. Adjustment was made for cell concentration in preparing samples for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Analysis of the LC-MS/MS data to determine peptide sequences followed by statistical filtering has shown that at least one 3T3 peptide biomarker exists for each biological cell group (vehicle control and three ricin dose levels). These peptides correspond to particular proteins expressed by the 3T3 cells. Identification of the relevant proteins, followed by metabolic pathway analysis to determine the pathways in which those proteins are involved, may reveal the survival mechanism for cells dosed at low levels, which in turn will provide insight into the related 3T3 physiological changes required to overcome ricin dosing.

Table 2. Statistical Filtering Results for Representative Peptides

Experimental Relative Molecular Mass	Calculated Relative Molecular Mass	Charge	Score[a]	Probability[b]	Peptide Sequence	Total Intensity	MS Scan	Biological Cell Group[c]	Replicate
1464.541	1462.815	2	4.78	0.95	IYVDDGLISLQVK	57490.9	4254.4254_2	VC	rep1
1567.444	1566.780	2	4.01	1.00	ITPSYVAFTPEGER	54813	3518.3518_2	IC50	rep1
1516.900	1515.749	3	3.94	0.22	IWHHTFYNELR	627427.4	3384.3384_3	VC	rep5
1321.153	1319.703	2	3.92	0.90	IMNTFSVVPSPK	32203.8	3671.3671_2	IC20	rep2
1171.348	1169.714	2	3.90	0.93	ILLAELEQLK	167745.2	4186.4186_2	IC20	rep4
1244.451	1243.653	2	3.65	1.00	IQLVEEELDR	212888.5	3517.3517_2	IC80	rep5
1269.171	1267.672	3	3.39	0.11	ISMPDVDLHLK	104355.8	3936.3936_3	VC	rep3
1259.407	1258.671	2	3.32	0.95	ISMPDIDLNLK	132932	4171.4171_2	IC20	rep6
1150.122	1148.579	2	3.08	0.76	ITESEEVVSR	18406.2	1985.1985_2	IC80	rep6
1275.388	1274.666	2	3.06	0.83	ISMPDIDLNLK	66842.3	3692.3692_2	IC20	rep5
1032.269	1031.498	2	3.01	1.00	IYFMAGSSR	15457.5	3104.3104_2	IC20	rep2
1198.183	1197.582	2	2.94	1.00	ITLDNAYMEK	102791.8	3230.3230_2	IC50	rep2
1317.295	1315.672	3	2.93	0.05	ISMPDFDLHLK	14825.9	4176.4176_3	IC80	rep4
1138.436	1136.599	2	2.89	0.69	IYGISFPDPK	51655	3916.3916_2	IC80	rep4
1214.381	1213.577	2	2.87	0.99	ITLDNAYMEK	6298.7	2919.2919_2	IC80	rep3
1391.869	1390.794	2	2.86	0.33	IPYDAKTIQTIK	19170.9	3374.3374_2	IC80	rep4
1284.264	1283.666	2	2.86	0.99	ISMPDVDLHLK	27798.5	3342.3342_2	IC50	rep1
948.442	947.498	2	2.79	0.97	ISQLEMAR	117440.6	2554.2554_2	IC80	rep4
1070.929	1069.571	3	2.78	0.06	ISMPDLHLK	63578.6	3018.3018_3	IC20	rep3
1541.671	1540.840	3	2.76	0.14	ISMPDIDLNLKGPK	25018.1	4019.4019_3	IC50	rep3
1157.456	1155.565	2	2.66	0.33	IYNMEMARK	31892.3	3658.3658_2	IC80	rep1
1075.286	1074.554	2	2.53	0.98	ITITNDQNR	35892.6	1681.1681_2	VC	rep6
1074.050	1073.584	2	2.51	0.84	IQEAGTEVVK	58600.9	2048.2048_2	VC	rep1
1088.240	1086.631	2	2.47	0.50	ITQVLHFTK	6852.2	3078.3078_2	VC	rep2
1259.741	1258.671	2	2.44	0.39	ISMPEVDLNLK	7492.1	4101.4101_2	IC20	rep1
1018.244	1017.569	2	2.43	0.99	ITITNDKGR	12178.3	1560.1560_2	VC	rep3
1284.715	1285.628	3	2.38	0.40	ISMPDVDLHMK	10488.7	5664.5664_3	VC	rep3
1293.169	1292.656	2	2.35	0.99	ISMPDFDLNLK	8644.8	4400.4400_2	VC	rep3
964.766	963.493	2	2.26	0.30	ISQLEMAR	3243.8	1692.1692_2	IC20	rep1

[a]The score indicates how well the observed spectrum matches the theoretical spectrum for a given peptide sequence.
[b]The probability indicates the likelihood that the peptide sequence is correctly determined based on the observed mass data.
[c]VC = vehicle control.

Table 3. Spectral Count Summary for Peptides with Total Spectral Count of 24 or Higher*

Peptide Sequence	Total Spectral Count	Spectral Count				Spectral Count % of Total			
		VC	IC_{20}	IC_{50}	IC_{80}	VC	IC_{20}	IC_{50}	IC_{80}
LVNELTEFAK	116	11	19	33	53	9	16	28	46
AGFAGDDAPR	115	43	42	10	20	37	37	9	17
YLYEIAR	89	13	17	20	39	15	19	22	44
HLVDEPQNLIK	62	9	12	16	25	15	19	26	40
YALYDATYETK	44	9	12	11	12	20	27	25	27
AEGPEVDVNLPK	43	9	14	10	10	21	33	23	23
QEYDESGPSIVHR	42	13	14	9	6	31	33	21	14
IQLVEEELDR	39	7	12	9	11	18	31	23	28
TLINILTER	39	8	12	11	8	21	31	28	21
RHPEYAVSVLLR	38	8	7	8	15	21	18	21	39
VSFELFADKVPK	38	17	11	4	6	45	29	11	16
IWHHTFYNELR	37	21	8	3	5	57	22	8	14
LGEYGFQNALIVR	37	10	9	8	10	27	24	22	27
VKGDVDVSLPK	37	10	9	10	8	27	24	27	22
VEIIANDQGNR	36	9	10	10	7	25	28	28	19
EGMNIVEAMER	35	8	7	10	10	23	20	29	29
HVGDLGNVTAGK	34	8	9	8	9	24	26	24	26
VSFELFADK	34	8	11	10	5	24	32	29	15
TLMNLGGLAVAR	32	10	10	7	5	31	31	22	16
QTALVELLK	31	6	8	10	7	19	26	32	23
DAGTIAGLNVLR	29	4	8	11	6	14	28	38	21
QLEDELVSLQK	28	7	9	8	4	25	32	29	14
APIIAVTR	27	8	9	6	4	30	33	22	15
IGFPWSEIR	27	9	11	5	2	33	41	19	7
LVTDLTK	27	4	4	6	13	15	15	22	48
GDVDVSLPK	26	6	6	6	8	23	23	23	31
KQTALVELLK	24	1	1	10	12	4	4	42	50
NDLAVVDVR	24	5	6	6	7	21	25	25	29
SGEIEPVSVK	24	5	7	8	4	21	29	33	17
SIDDLEEK	24	9	6	5	4	38	25	21	17
VLQATVVAVGSGGK	24	4	7	6	7	17	29	25	29

*Yellow highlight: spectral count percentage for the peptide is at least 10 percentage points higher for the highlighted biological cell group than for the other biological cell groups.

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